**Cell-Mediated Drug Delivery as a Treatment to Inhibit Restenosis**

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**Abstract**

Cardiovascular diseases (CVDs) are the leading cause of death in the world. CVDs are typically due to atherosclerosis, a build-up of plaque that results in arterial blockage. Currently, the most common solution is balloon angioplasty surgery, which is only a temporary solution because even though the arteries are widened again, revascularization results in arterial injury. This injury triggers the body's natural response: inflammation through the proliferation and migration of smooth muscle cells (SMC), narrowing the artery again—a process called restenosis—requiring further surgeries. Cinnamic aldehyde (CA) has been shown to inhibit SMC proliferation and migration by activating the Nrf2-Keap1 pathway, but it has a systemic effect. We aim to form a cell-mediated targeted drug delivery system using macrophages and micelle nanoparticles to transport CA to activate the Nrf2-Keap1 pathway and prevent restenosis. As a result of sparse data, the micelles were first characterized by dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) to determine size (232.7 nm in diameter) to ensure the nanoparticles were large enough to be taken up by macrophages. Then, THP-1 macrophage uptake of rhodamine labeled micelles was studied by live-cell microscopy. Through Western Blot and 3-(4,5-Dimethylthiazol-2-Yl)- 2,5-Diphenyltetrazolium Bromide (MTT) assays, CA-loaded nanoparticles were found to activate the Nrf2-Keap1 pathway and inhibit vascular SMC proliferation, respectively. These results establish the groundwork for future investigation into pluronic micellar targeted delivery to prevent restenosis.

## Introduction

Responsible for over thirty percent of deaths in the world, cardiovascular diseases (CVDs) pose a serious threat.[7] Atherosclerosis is the underlying cause of most CVDs. Atherosclerosis is the accumulation of fat deposits in the arteries, resulting in plaque build-up and blockage of blood flow, which can lead to coronary heart disease, strokes, and other heart and blood vessel disorders. Current revascularization methods, including balloon angioplasty, stenting, and bypass grafting, are only effective temporarily: even though surgeries widen arteries, the body registers any type of intervention as injury and responds by inflaming the affected site through the proliferation and migration of vascular smooth muscle cells (VSMCs). Driven by cell proliferation, the arterial wall thickens, and the artery narrows again in a process called restenosis, warranting further surgeries. These surgeries are expensive, and as a consequence, resources are wasted. Researchers are investigating ways to prevent restenosis by impeding VSMC proliferation and migration. As VSMC proliferation and migration is mediated by increased production of reactive oxygen species (ROS) in CVDs, we explored an avenue of altering ROS levels.

One approach to reduce VSMC proliferation by altering the levels of ROS is the Nrf2-Keap1 pathway. Keap1 regulates the activity of Nrf2 (NF-E2-related factor 2)—an inducible transcription factor. In normal conditions, Nrf2 is degraded, but under oxidative or electrophilic stress, Nrf2 is not degraded and translocates to the nucleus. This allows Nrf2 to promote the expression of antioxidant enzymes.[6] The Bahnson Research Group has identified cinnamic aldehyde (CA), the hydrophobic compound responsible for the unique flavor and scent of cinnamon, as capable of activating Nrf2 by its electrophilic nature and inhibiting VSMC proliferation and migration.[1] By activating the Nrf2-Keap1 pathway, CA intensifies antioxidant

production and effectively reduces the proliferation and migration of VSMCs. However, CA has a systemic effect, reducing VSMC proliferation and migration throughout the body. Therefore, we aim to develop a cell-mediated targeted delivery system that will activate the Nrf2-Keap1 pathway and prevent restenosis.

Since part of the body's natural arterial injury response involves the migration of macrophages to the site of injury, macrophages can act as transport vehicles.[2,3,8] We propose packaging CA in pluronic micelles (PMs) to increase macrophage uptake, and use the macrophages to deliver CA to VSMCs. PMs are tri-block copolymers, with two hydrophilic blocks surrounding a hydrophobic block. The lengths of these polymers vary among pluronics, resulting in differing hydrophobicities. At concentrations above the critical micellar concentration (CMC), PMs are in a dynamic equilibrium between assembling into micelles and disassembling into separate molecules. Because of this dynamic equilibrium and CA's hydrophobicity, we know CA will be encapsulated when the PMs form and released when they disassemble. Therefore, we hypothesize that CA-encapsulated PMs will be taken up by macrophages and then delivered and released to VSMCs to inhibit proliferation locally. To test this hypothesis, we characterized pluronics, observed THP-1 macrophage uptake, and treated Sprague Dawley cells *in vitro* to confirm inhibition of VSMC proliferation and Nrf2-Keap1 pathway activation.

**Methods**

**Cell Culture** Vascular smooth muscle cells (VSMCs) from Sprague Dawley rats were stored at concentrations of $1x10^6$ cells/mL in cryovials in 90% complete media (DMEM and Ham's F-12 in a 1:1 ratio with 10% FBS, 1% streptomycin/penicillin, and 0.8% L-glutamine) and 10% DMSO in liquid nitrogen tanks. $1x10^6$ cells were thawed into a 175 $cm^2$ Corning® Cell Culture

Flasks with fresh complete media to adhere and grow until reaching 80% confluency before use. Once a week, when cells reached 80% confluency, the VSMCs were passed into new flasks at a density of $1x10^6$ cells/flask in complete media until the tenth passage, at which point they were discarded.

THP-1 monocytes in RPMI media with 10% FBS and 1% streptomycin/penicillin differentiated after being in the presence of 162 nM PMA for 48 hours. Differentiation was confirmed by assessment of cell morphology by light microscopy and then THP-1 macrophages were stored in 75 $cm^2$ Corning® Cell Culture Flasks.

**Pluronic Micelles** Solid pluronics F-127 (75.5% ethylene oxide and 24.5% propylene oxide, 12,600 g/mol) and P-84 (46.9% ethylene oxide and 53.1% propylene oxide, 4,200 g/mol) were dissolved in distilled water to form 20% mass by volume solutions, and solid pluronic L-101 (23.4% ethylene oxide and 76.6% propylene oxide, 3,800 g/mol) was dissolved in distilled water to form a 15% mass by volume solution. The solutions were stored in $4^{\circ}C$ until use.

**Critical Micellar Concentration (CMC) Determination** A BioTek® Cytation 5 Cell Imaging Multi-Mode Reader was used to determine fluorescence emission maxima of 300 nM Nile Red solutions from wavelengths 575 nm to 700 nm with varying concentrations of pluronic from 0% to 20% mass by volume. These measurements were taken in triplicates. The average differences and standard deviations in peak fluorescence between the pure solvent and each of the other solutions containing pluronic in greater than 0% mass by volume were calculated and graphed with the OriginLab® software OriginPro 2016 to identify the concentration at which micelles formed for each pluronic.

**Dynamic Light Scattering (DLS)** The Malvern Zetasizer Nano series program quantified the sizes and level of dispersion of micelles diluted to 0.025% mass by volume in distilled water and PBS in glass Malvern cuvettes.

**Nanoparticle Tracking Analysis (NTA)** A Nanosight NTA was primed with filtered, distilled water for quantifying the size and size distribution of nanoparticles (PMs in this instance) diluted to 0.025% in distilled water, and then primed with filtered PBS to quantify the size and level of dispersion of micelle sizes diluted 0.025% mass by volume in PBS.

**Fluorescent Labeling of PMs** Pluronics were labeled with 5.25 μM rhodamine-123 (Sigma Cat#83701) to track PMs via fluorescence microscopy using a GFP filter (ex=450-480nm, em=500-550nm; recommended ex=488nm, em=515-575nm). For some experiments, THP-1 macrophages were grown on a cover slip, labeled with 0.05 μM, 0.1 μM, and 0.5 μM DiI, and incubated at 37°C for 18 hours. After incubation, the coverslips were dipped in 6 mM DAPI to label the macrophage nuclei.

**Macrophage Micelle Uptake** Bright-field and fluorescence microscopy was accomplished with the BioTek® Cytation 5 Cell Imaging Multi-Mode Reader to observe the uptake of 5.25 μM rhodamine-123-labeled micelles by THP-1 macrophages over the course of 4 hours. Macrophage uptake was compared to uptake of rhodamine-123 without micelles, along with macrophage uptake of CA-encapsulated micelles. Images of fluorescently labeled pluronics and macrophages were also taken with a Zeiss microscope using a 60X objective to confirm macrophage uptake of rhodamine-123-labeled PMs, using DAPI, Cy3, and Cy5 filters to detect.

**MTT** Sprague Dawley VSMC were seeded onto a Falcon® 96-Well Plate at a density of $5x10^3$ cells/well. VSMC were treated with varying concentrations of just CA (0 μM to 1.6 mM), just pluronic L-101 (from 0.01% to 10% mass by volume), and finally, CA encapsulated in a constant

concentration of 0.05% pluronic L-101 (0 μM to 800 μM) in replicates of eight. After treatment, a solution of 0.4 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye) and VSMC complete media was administered in equal concentrations to all conditions and the cells were incubated for four hours before the dye solution was dumped and left to dry. After 24 hours, DMSO was added to the dry wells and the BioTek® Cytation 5 Cell Imaging Multi-Mode Reader indirectly quantified the concentration of living cells based on absorbance values. The experiment was repeated three times.

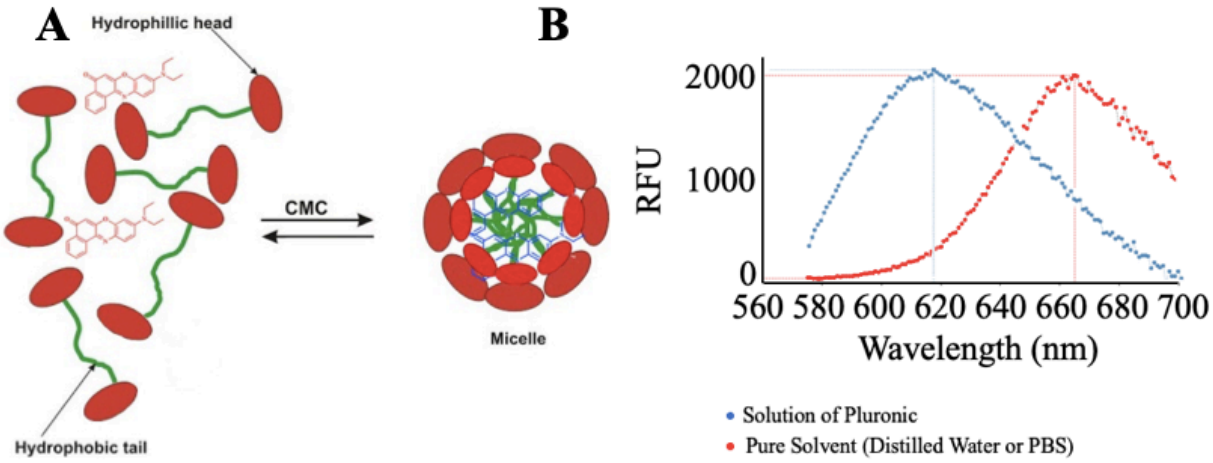
**Western Blot** Sprague Dawley VSMC were plated at $1.5x10^6$ cells in a 10 cm petri dish and treated with four conditions in triplicate for four hours: VSMC complete media, 0.05% pluronic L-101, 100 μM CA, and 0.05% pluronic L-101 with 100 μM CA. The dishes were rinsed twice with HBSS and placed in ice for 10 minutes. The cells were collected into separate centrifuge tubes using a cell lifter. The cells were centrifuged for 5 minutes at 1200 rpm. The HBSS was replaced with 1X Lysis Buffer. Heat-denatured proteins were added in equal concentrations (quantified by using Pierce™ BCA Protein Assay Kit and protocol) to the lanes of a nitrocellulose gel with SDS and gel electrophoresis was run for 1 hour at a constant voltage of 180 V in 1X Running Buffer. The proteins were transferred to a PVDF membrane for 2 hours at a constant current of 250 mA. The membrane was submerged in blocking buffer overnight, rinsed with PBS-T, and blotted with primary antibodies targeting Nrf2, HO-1, and ß-actin, and secondary fluorescent antibodies. The membrane was imaged with LI-COR® Odyssey Imaging.

**Results**

Micelle Formation

Pluronics are tri-block co-polymers with hydrophobic blocks at both ends of hydrophilic blocks. When dissolved to form an aqueous solution, the block co-polymer seeks to minimize

interactions between the hydrophobic block and water. Therefore, at high enough concentrations, the block co-polymers naturally assemble to form micelles, in which the hydrophobic polymers are internalized and separated from water by the hydrophilic polymers. These micelles are in dynamic equilibrium between micelle nanoparticles and dissociated free polymers. The lowest concentration at which the micelles form is called the critical micelle concentration (CMC). To determine the CMCs of each pluronic, a Nile Red fluorescence assay was conducted, in which the fluorescent emission maxima of Nile Red depended on the polarity of the environment experienced. In polar environments, Nile Red maximally fluoresces red, but in apolar

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environments, Nile Red fluorescent emission maxima shift toward lower wavelengths, known as blue shifts. The fluorescence emission maxima of 30 µM Nile Red in serial dilutions of the three

CMC = 4%
CMC = 8%

CMC = 8%
CMC = 8%

CMC = 0.01%
CMC = 0.01%

PBS
Water

Figure 1. **Determination of CMC.** (A) Mechanism of Nile Red fluorescence to identify CMC. (B) Peak fluorescent intensity of pure solvent against different PM solutions of varying concentrations with Nile Red was measured. (C) Blue shifts between the pure solvent and each concentration of the three PMs identify varying or high CMCs for both F-127 and P-84, and a low CMC of 0.01% for L-101 (n = 3). Error bars represent standard deviation.

different PMs (F-127, P-84, and L-101) in water and PBS were measured. These intensities were used to identify the CMCs through calculating the blue shift—the difference in peaks of fluorescence between a pure solvent and a solution of the solvent with pluronic (Fig 1a). The blue shift was similar for the dilutions at which micelles did not form, and then increased greatly to similar shifts for concentrations at which micelles did form, in which they encapsulated the fluorophore Nile Red and affected its maximal emission wavelength. Based on the concentrations of pluronic at which jumps in the blue shifts occurred, it was determined that the CMC was 8% in water and 4% in PBS for Pluronic F-127 (PF127), 8% in both water and PBS for Pluronic P-84 (PP84), and 0.01% in both water and PBS for Pluronic L-101 (PL101) (Fig 1b). Pluronic L-101 had the lowest CMC, hence we chose to study this polymer further.

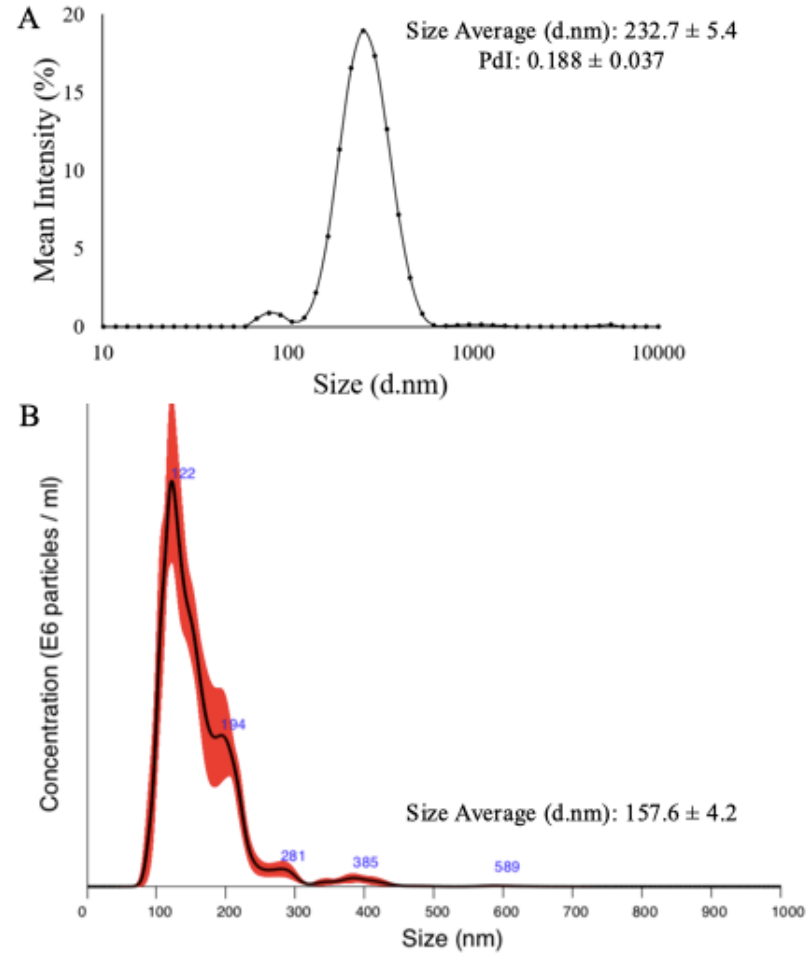


Figure 2. **Characterization of P-L101.** (A) The size and level of dispersion was measured by DLS. (B) NTA also measured size and dispersity, in addition to the concentration of particles.

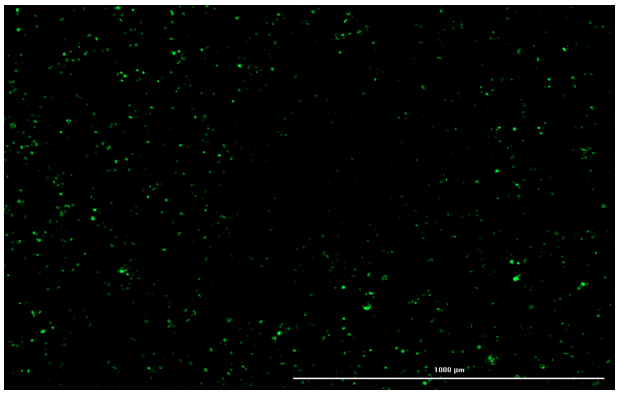

Figure 3. **Rhodamine effectively labels P-L101 PMs.** The efficacy of rhodamine uptake by Pluronic L-101 4 hours after rhodamine

We used two methods to determine the size of Pluronic L-101 PMs. Dynamic Light Scattering (DLS) determined the average size (250.9 nm in diameter) and polydispersity (0.193) of a 0.025% filtered solution of PMs in water (Fig 2a). Nanoparticle Tracking Analysis (NTA)

also measured the average size of a filtered 0.025% solution of PMs in water (158.1 nm in diameter) (Fig 2b).

To label the Pluronic L-101 PMs, we examined the uptake the green fluorophore rhodamine-123 for future imaging experiments in which we needed to differentiate the PMs from cells. These PMs were very effective, as indicated by the contrast between the bright green fluorescent in the micelles and the dark background. (Fig 3).

THP-1 Macrophage Uptake of PMs

The ability of PMA-induced THP-1 macrophages (Figure 4), to uptake PMs was assessed by manually imaging cells every 15 minutes for 4 hours. Three conditions were tested: 1) a rhodamine control to confirm that fluorescence does not enter the macrophage without the aid of a pluronic (Fig 5a), 2) the rhodamine-labeled PMs administered alone (Fig 5b), and 3) rhodamine-labeled PMs with 50μM of cinnamic aldehyde (Fig 5c). Images of the rhodamine control show that without the pluronic, macrophages do not uptake rhodamine since the macrophages do not fluoresce green after four hours (Fig 5a). Contrasting the pictures taken

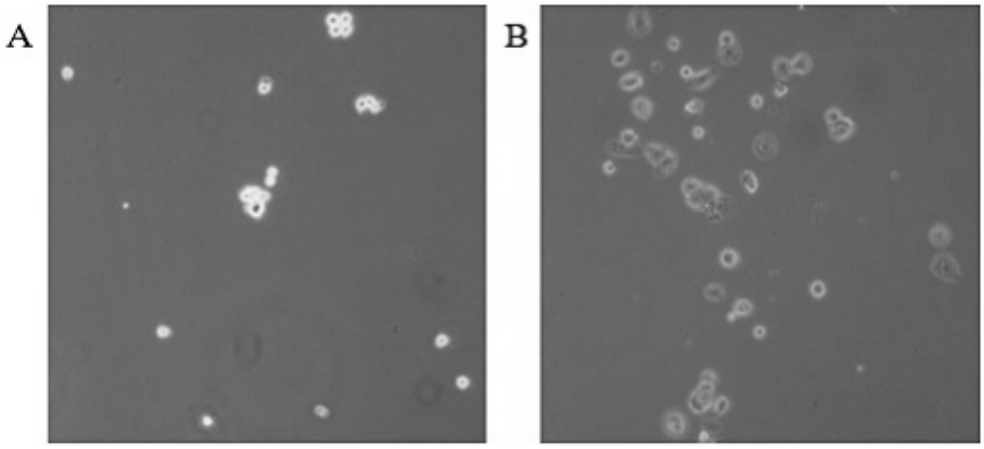


Figure 4. **THP-1 Macrophage differentiation with PMA.** Macrophages imaged with a 10X objective (A) before and (B) after 24 hours of differentiation in PMA.

of the macrophages incubated with only rhodamine and the pictures taken of the macrophages incubated with rhodamine-123-labeled PMs and rhodamine-123-labeled PMs with cinnamic aldehyde, the macrophages fluoresce green after four hours. This indicates that the macrophages take no more than 4 hours to uptake the PMs, represented by the macrophages fluorescing green after four hours when PMs are present, but not fluorescing when PMs are absent (Fig 5b, c). Any green observed in the images taken immediately after treatment is due entirely to the green background filter and does not indicate macrophage uptake.

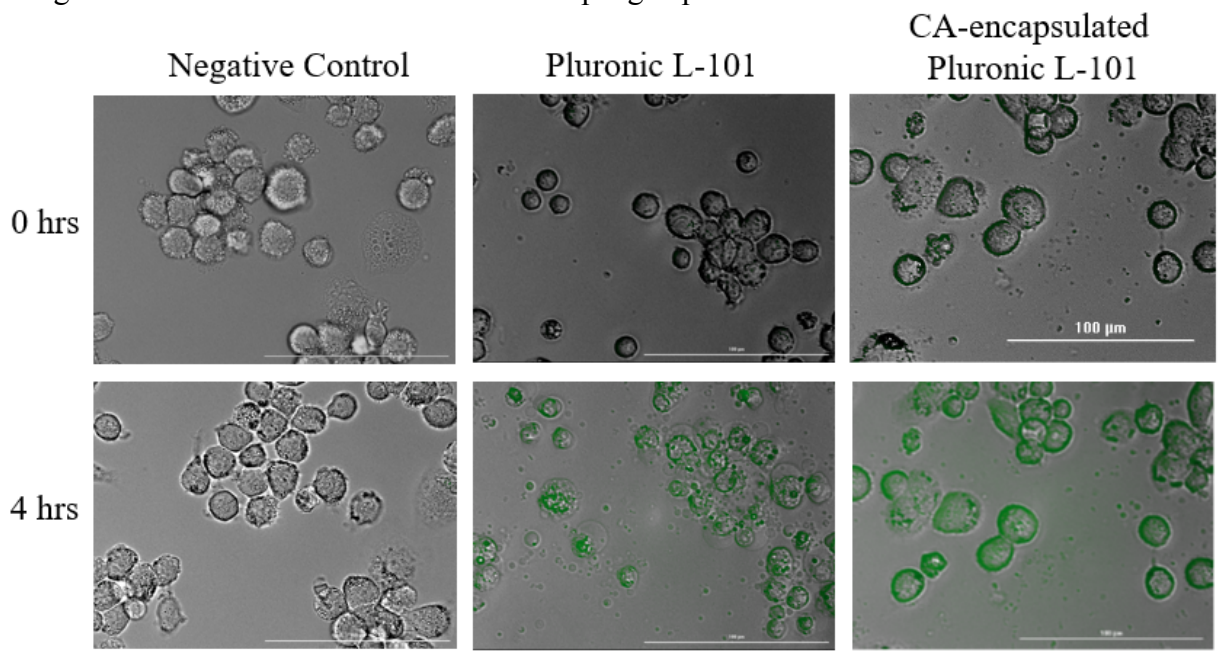


Figure 5. **THP-1 Macrophage Uptake of CA-PMs.** Top panels show live imaging of THP-1 macrophages immediately after treatment with rhodamine-123 alone, rhodamine-123-labeled PMs, or rhodamine-123-labeled CA-PMs, and the bottom panels show live imaging of THP-1 macrophages 4 hours after treatment.

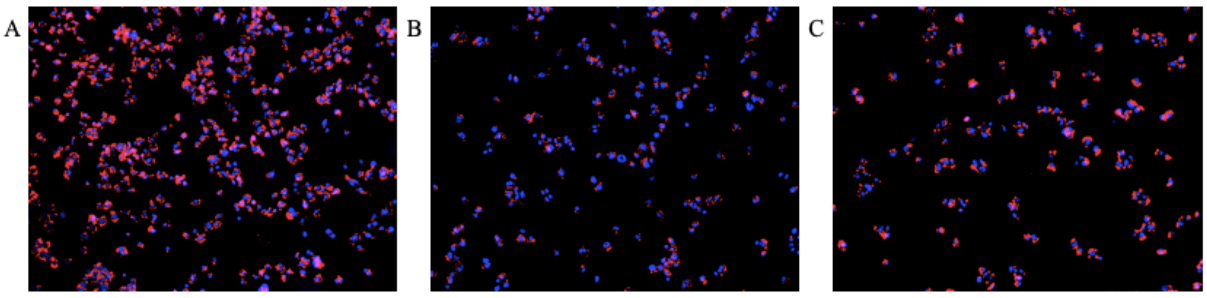


Figure 6. **Labeling THP-1 Macrophages with DiI.** Macrophage uptake of fluorescent maximized with (a) 0.5 µM DiI after 24 hours, (b) 1 µM after 6 hours, and (c) 1 µM after 3 hours.

THP-1 Macrophage Labeling with DiI

Differentiated THP-1 monocytes were incubated in duplicates with either 0.5 μM or 1.0 μM fluorescent DiI for 24 hours (Fig 6a), 6 hours (Fig 6b), and 3 hours (Fig 6c). When incubated for 24 hours, the best uptake was of 0.5 μM DiI, but for the other two time points, 1.0 μM DiI yielded the best results. To confirm that the DiI observed was in fact taken up by macrophages, the macrophage nuclei were stained with DAPI prior to imaging. Having confirmed that macrophages could be labeled with DiI, macrophage uptake of DiI was further examined to determine if it was possible to label the macrophages with lower concentrations of DiI (0.1 and 0.05 μM) at a longer incubation time (18 hours). We observed high levels of macrophage labeling with 0.05 μM DiI and 18 h incubation (Fig 6). Finally, we assessed the uptake of rhodamine-123-labeled Pluronic L-101 PMs and cinnamic aldehyde-encapsulated in Pluronic L-101 PMs tagged with rhodamine by DiI-labeled macrophages. This double-staining technique would allow for future experiments where we will use two different cell types in co-culture. We confirmed the presence of rhodamine-123-labeled L101 PMs in DiI-labeled macrophages after 18-hour incubation (Fig 7).

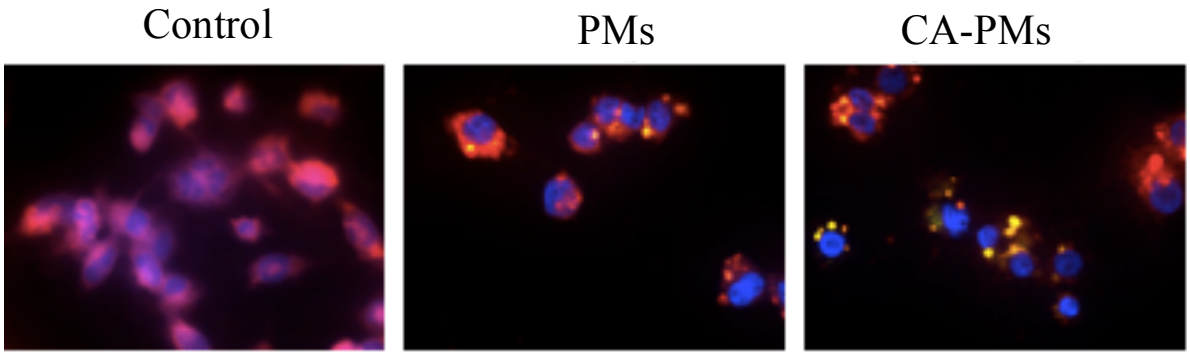


Figure 7. **Labeled THP-1 Macrophage uptake of labeled PMs.** THP-1 Macrophages labeled with DiI for were incubated with rhodamine-labeled PMs and rhodamine-labeled CA-PMs for 24 hours and imaged with an epifluorescence microscope (63x objective lens). THP-1 macrophages taking up rhodamine-labeled PMs are visualized by DiI positive puncta (yellow).

Free and Encapsulated CA Inhibits Cell Proliferation

MTT assays were run in triplicate to identify the individual effects of PMs and cinnamic aldehyde on Sprague Dawley rat aortic smooth muscle cells, and then the combined effects of these two treatments. PMs alone had an EC50 of 0.12% mass by volume (Fig 8a), meaning that at this concentration, VSMCs experienced half-maximal toxic effects. Therefore, in order to form micelles for CA encapsulation, but also not cause more than half-maximal toxic effects, Pluronic L101 PMs can only be administered in concentrations between 0.01% and 0.12% mass by volume. In the trials testing different concentrations of just CA, the EC50 was found to be 250 µM (Fig 8b), and to assess if encapsulation in PMs affects the CA EC50, an MTT assay was run with a constant concentration of PMs at 0.05% mass by volume and varying concentrations of CA. The PM concentration was chosen to be 0.05% because it was a concentration above the CMC but below the EC50, such that the micelles should not affect the VSMCs. From this assay, encapsulation of CA appears to cause a shift in the EC50 to 430 µM (Fig 8c). This could be due to the cells only being incubated with PMs containing CA for four hours and CA contained by the PMs was unable to inhibit cell proliferation. Therefore, CA release from the PMs would be necessary for CA to inhibit cell proliferation.

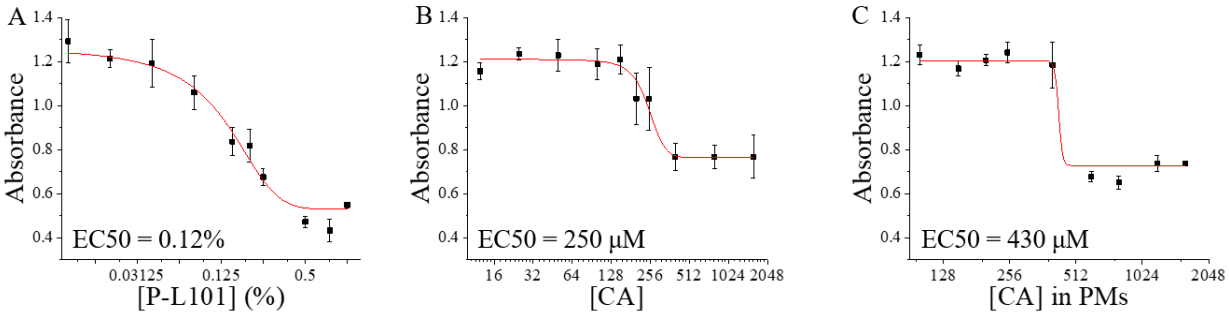


Figure 8. **Encapsulation of CA in PMs Increases its EC50 in VSMC.** Cell viability at different concentrations of (a) Pluronic L-101, (b) cinnamic aldehyde, and (c) cinnamic aldehyde with a constant Pluronic L-101 concentration were measured by an MTT assay (n=24). Error bars represent standard deviation.

CA-Dependent Activation of the Nrf2 Pathway

Activation of the Nrf2 pathway requires stabilization and accumulation of the Nrf2 protein. Our lab has previously shown that CA is capable of activating the Nrf2 pathway in VSMC due to its electrophilic nature.[1] For PMs to be an effective delivery vehicle for CA, we tested if encapsulated CA was capable of inducing Nrf2. VSMCs were treated with media, empty PMs, free CA (250 $\mu$M), or CA-PMs (250 $\mu$M CA in 0.05% PMs) for 8 hours to be used in Western blot assays performed in triplicates. Cells were collected and immunoblot for Nrf2 was performed. The Nrf2 pathway was activated only when CA was present (empty PMs alone did not activate the Nrf2 pathway), and even when encapsulated in PMs, CA was still found to activate the Nrf2 pathway (Figure 9).

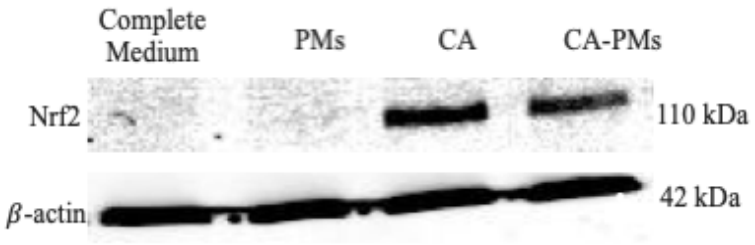


Figure 9. **Encapsulated CA Activates Nrf2.** Western blot analysis of Nrf2 and β-actin protein production under the following conditions: complete VSMC media, 0.05% PMs, 250 $\mu$M CA, and 0.05% PMs with 250 $\mu$M CA (left to right) for 8 hours.

**Discussion and Conclusions**

After determining the CMC of three pluronics (F127, P84, and L101), we selected to work with P-L101 because it had the lowest CMC. This will allow us to use lower concentrations of polymer and achieve encapsulation of our drug of interest, CA. There were no significant differences in CMC when the assay was performed in water or PBS. This pluronic, P-L101 formed PMs, with a diameter of 232.7 nm and a polydispersity index of 0.188 based on DLS readings. NTA readings were smaller by an average of 75.1 nm, but it may have been due to the NTA analyzing noise from other particles, rather than just PMs. We successfully labeled the PMs with rhodamine-123 to allow for fluorescent imaging. Uptake experiments show that macrophages can take up the PMs as evidenced in Fig 4 and Fig 7, and that uptake is maximal within 4 hours. Based on the MTT assays, PMs do not inhibit VSMC proliferation at the CMC, which allows us to use them as delivery vehicles for CA. CA has been shown to also inhibit the proliferation of VSMCs[1], skin cells[9], and cancer cells, such as in the liver[5] and colon[4].

Delivering CA in micelles was expected to change the EC50, with the original hypothesis that the EC50 was going to decrease. Unexpectedly, PMs caused an increase in the EC50 of cinnamic aldehyde by almost 200 μM. This may be explained by a slow release of CA from the micelles when they disassemble into dissociated polymers due to their dynamic equilibrium. More experiments are needed to determine the mechanism by which PMs increase the EC50 of CA in VSMC. We also found that encapsulation of CA in PMs did not ultimately inhibit CA's ability to activate Nrf2 and that such activation was not due to the presence of the PMs based on the Western blot. We found that it took eight hours for CA in PMs to maximize Nrf2 protein production, while CA alone took four hours to maximize Nrf2 protein production, which supports the theory of slow CA release by PMs.

Together, these results indicate that PMs can be loaded with CA to be taken up by macrophages and that the released CA-encapsulated PMs will activate the Nrf2-Keap1 pathway, inhibiting the proliferation of VSMCs and therefore inflammation, thus preventing restenosis. There are limitations to the research conducted thus far that require further experimentation to confirm results. The MTT assays are an indirect measurement of cell viability, showing only the relative number of living VSMCs, but do not quantify the ratio of live VSMCs to dead VSMCs. Furthermore, a co-culture has yet to be conducted, so it is currently unknown if the macrophages can deliver the micelles to the VSMCs, and if they can, how the VSMCs are affected. Therefore, future directions include using the Muse® Cell Viability Assay to determine cell count of living and dead cells, in addition to forming, observing, and quantifying a co-culture of VSMCs, THP-1 macrophages, and CA-encapsulated PMs. Visually showing the release by macrophages and uptake by VSMCs of CA-PMs would be a step towards making cardiovascular surgery a more permanent solution.

**Acknowledgements**

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